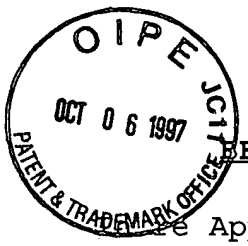
GP 1804

210/048
Patent



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:	)	Group Art Unit: 1804
	)	
Gjerset and Sobol	)	Examiner: Low, C.
	)	
Serial No.: 08/335,461	)	
	)	
Filed: November 7, 1994	)	
	)	
For: ENHANCING THE SENSITIVITY OF TUMOR CELLS TO THERAPIES	)	

TRANSMITTAL LETTER

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith for filing in the above-referenced application are the following:

- Reply Brief (in triplicate);

CERTIFICATE OF MAILING

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

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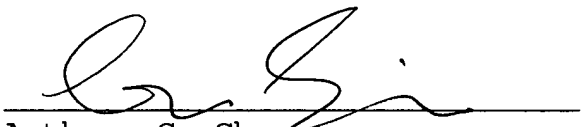
- Copies of:
  . Shimizu et al., Oncogene 9:2441-2448 (1994)
  . Stone et al., Cancer Res. 56:3199-3202 (1996); and

- Return postcard.

No fee is due with this filing but if additional fees are required, please charge our Deposit Account No. 12-22475 for the appropriate amount..

Respectfully submitted,

LYON & LYON LLP



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of Ruth Gjerset and Robert E. Sobol
United States Patent Application Serial No. 08/355,461
Filed November 7, 1994

REPLY BRIEF FILED PURSUANT TO 37 C.F.R. § 1.193 (b)

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CITED REFERENCES

Pursuant to 37 C.F.R. § 1.193(b), Applicant hereby replies to a new point of argument raised in the Examiner's Answer mailed August 4, 1997. If any fee is required with this filing, please charge our Deposit Account No. 12-2475 for the appropriate amount.

NEW POINT OF ARGUMENT

In the Examiner's Answer, starting at line 24 of page 18 and continuing on to line 24 of page 19, the Examiner raised a new point of argument, i.e., reduction of tumor burden.

According to the Examiner, because a wild-type p53 gene suppresses tumor growth, it alters the tumorigenic phenotype of a transfected cancer cell to that of a "normal" cell and results in fewer cells with a tumorigenic phenotype. In other words, the transfection of a wild-type p53 gene to tumor cells reduces the tumor burden of a patient. Routine cancer therapies such as chemotherapies and radiation therapy also reduce tumor burden by killing tumor cells. Because a wild-type p53 gene effectively alters the transfected tumor cells to "normal" cells, the Examiner alleged, the **remaining** nontransfected tumor cells are more easily treated by routine cancer therapies such as chemotherapies and radiation therapy. As such, the Examiner argued, it would have

been obvious for one skilled in the art to combine a routine cancer therapy with the transfection of tumor cells with a wild-type p53 gene.

Applicant respectfully traverses this new point of argument by the Examiner.

REPLY

I. THE ARGUMENT OF "REDUCTION OF TUMOR BURDEN" DOES NOT APPLY TO THE CLAIMED INVENTION BECAUSE THE PENDING CLAIMS ARE DIRECTED TO TREATING TUMOR CELLS WITH AN EXOGENOUS WILD-TYPE p53 GENE

The Examiner's argument about reducing tumor burden by (1) using a heterologous wild-type p53 gene to treat some cancer cells and (2) using a routine cancer therapy to treat the remaining cancer cells with no heterologous genetic material not only does not apply to the claimed invention, but also teaches away from the claimed invention.

Claim 1 of the present application describes a method of increasing the therapeutic effect of a cancer therapy by "delivering a wild-type p53 gene to a tumor cell which is deficient in its wild-type p53 gene, effecting the expression of said wild-type p53 gene in **said** tumor cell, and subjecting said tumor cell to **said** cancer therapy." Unlike the scenario described by the Examiner where a cancer therapy is directed to cancer cells with no

heterologous genetic material, the method of claim 1 is directed to treating the tumor cell transfected with a wild-type p53 gene with the cancer therapy. In other words, a tumor cell is given a heterologous wild-type p53 gene, and thereafter the **same** tumor cell is treated with a cancer therapy.

Contrary to claim 1 of the pending application, the Examiner stated at line 13, page 19 of the Examiner's Answer that "[c]ancer therapy is not directed to killing 'normal' cells" containing a heterologous wild-type p53 gene. Therefore, one skilled in the art would have been taught away from the claimed invention by the theory of "reduction of tumor burden."

II. IT WOULD NOT HAVE BEEN OBVIOUS THAT A GENE THAT SUPPRESSES TUMOR GROWTH WOULD SENSITIZE TUMOR CELLS TO ROUTINE CANCER THERAPIES SUCH AS CHEMOTHERAPY

In making the argument of "reduction of tumor burden," the Examiner ignored differences between two cancer treatment modalities:

(1) suppressing tumor growth by the expression of a heterologous tumor suppressor gene; and

(2) increasing the sensitivity of a tumor cell to a chemotherapeutic agent (or other routine cancer

therapies) by the expression of a heterologous therapy sensitizing gene.

Suppression of tumor growth cannot be equated with enhancement of tumor sensitivity to chemotherapeutic agents and radiation therapy. In fact, it is commonly known in the prior art that nondividing cells and slow-growing cells tend to be less susceptible to chemotherapy and radiation therapy than fast-growing tumor cells. As the Examiner stated at line 13, page 19 of the Examiner's Answer, "[c]ancer therapy is not directed to killing 'normal' cells." Research papers have shown that some tumor suppressor genes actually decrease the sensitivity of tumor cells to chemotherapy.

For example, two tumor suppressor genes, Rb and p16, have been shown to increase tumor cells' resistance to cisplatin and other chemotherapeutic agents. Shimizu et al., Oncogene 9:2441-2448 (1994) observed that the stable expression of a wild-type Rb gene after its transfection into a small lung cancer cell line resulted in increased resistance of the cancer cells to cisplatin, etoposide and doxorubicin. Stone et al., Cancer Res. 56:3199-3202 (1996) observed that the induced expression of a heterologous wild-type p16 gene in a melanoma cell line confers the melanoma cells greater resistance to chemotherapeutic agents cisplatin,

vinblastine and methotrexate. These two papers are attached with this reply brief for the reference of the Board of Patent Appeals and Interferences and the Examiner.

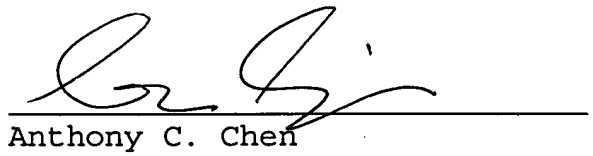
These two references support that Applicant's discovery that expressing a heterologous wild-type p53 gene in a tumor cell before subjecting the tumor cell to a cancer therapy increases the therapeutic effect of the therapy is an unexpected result.

CONCLUSION

In view of the above discussion, Applicant submits that claims 1, 2, 4-20 and 23 are allowable. Applicant respectfully requests that they be allowed and passed to issue.

Respectfully submitted,

LYON & LYON LLP



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Oncogene (1994), 9, 2441–2448

RB protein status and clinical correlation from 171 cell lines representing lung cancer, extrapulmonary small cell carcinoma, and mesothelioma

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We have studied RB protein expression in 171 cell lines derived from patients with small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), pulmonary carcinoid, mesothelioma, and extrapulmonary small cell cancer (EPSC) and have correlated this data with clinical outcome. We detected absent or aberrant RB protein expression in 66/75 SCLC, 12/80 NSCLC, 1/6 carcinoid, 0/5 mesothelioma, and 4/5 EPSC samples. In addition, we observed integration of human papilloma virus (HPV) DNA in the single EPSC cell line that retained wildtype RB protein. We did not detect integration of HPV, SV40 or adenoviral DNA in other tumor samples with wildtype RB status. We also noted a stable, hypophosphorylated mutant RB in 12 SCLC and 3 NSCLC samples which might have been falsely interpreted as wildtype by current immunohistochemical techniques. Analysis of the matched clinical data showed no associations between RB status and age, sex, extent of disease, performance status, smoking history, and previous treatment. In addition, retrospective analyses showed no consistent correlation of RB protein expression with either best clinical response, overall survival, or *in vitro* chemotherapeutic drug sensitivity. The stable expression of RB after gene transfection into RB(−) SCLC cells, however, resulted in a trend toward increased *in vitro* resistance to etoposide, cisplatin and doxorubicin.

Introduction

Mutational inactivation of the *Rb* tumor suppressor gene has been detected in 100% of familial and sporadic retinoblastoma tumors (Knudson, 1971; Weinberg, 1991). Other human malignancies, however, have also been associated with *Rb* inactivation including sarcomas, hematopoietic tumors, and carcinomas of the lung, breast, bladder, prostate and others (Friend *et al.*, 1987; Harbour *et al.*, 1988; T'Ang *et al.*, 1988; Bookstein *et al.*, 1990; Cheng *et al.*, 1990; Horowitz *et al.*, 1990). Although involvement of the *Rb* gene in common adult tumors was initially unexpected, the recent recognition that the activity of the *Rb* gene product (RB) is modulated by members of the cyclin dependent kinase family (Sherr, 1993) and that RB may function to regulate general transcription factors (Nevins, 1992) has suggested important growth regulatory roles for this protein in tissues of varying lineages.

One of the first non-retinoblastoma tumors to be associated with *Rb* inactivation was small cell lung cancer (SCLC), a tobacco-related malignancy that constitutes approximately 25% of all cases of lung cancer in the United States (Boring *et al.*, 1993). Prompted by cytogenetic (Harbour *et al.*, 1988) and restriction fragment length polymorphism (Yokota *et al.*, 1987) data, we initially observed abnormalities in the structure and expression of the *Rb* gene in a subset of SCLC cell lines using DNA and RNA blot analysis (Harbour *et al.*, 1988). Subsequently, additional SCLC samples with normal appearing *Rb* mRNA were shown to have either undetectable or aberrant expression of the RB product (Yokota *et al.*, 1988; Hensel *et al.*, 1990; Horowitz *et al.*, 1990; Kaye *et al.*, 1990), suggesting that many SCLC samples had inactivated RB activity. Other studies, however, using derived cell lines and tumor xenografts have reported a lower frequency of RB inactivation in SCLC (Rygaard *et al.*, 1990).

In contrast to SCLC, only a small subset of non-small cell lung cancer (NSCLC) tumor samples had evidence for *Rb* inactivation by DNA and RNA blot analyses (Harbour *et al.*, 1988). This lower incidence of RB abnormalities more closely resembled the frequency observed in other tumor types including carcinomas of the breast, bladder and prostate (Horowitz *et al.*, 1990). Recently, however, Xu and colleagues reported an abnormal RB protein staining pattern in 10 of 34 primary NSCLC tumors using immunohistochemical techniques. They also observed that advanced-stage NSCLC had a higher frequency of altered RB protein expression than low-stage tumors (Xu *et al.*, 1991a). Another analysis of NSCLC primary tumor samples collected from the Lung Cancer Study Group demonstrated abnormal RB immunohistochemistry staining in 32% of their samples. In addition, this study analysed clinical outcome, however they did not detect any correlation of RB status with either time to relapse or overall survival in their matched patient samples (Reissman *et al.*, 1993). These authors also noted the enhanced sensitivity of protein analysis as compared to DNA and RNA blot analysis, however, their use of immunohistochemical techniques also presented several limitations that might result in false positive and false negative readings. These include: (1) reactivity of commercially available antisera with mutant RB products

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Received 11 March 1994; accepted in revised form 25 May 1994

which are incorrectly interpreted as wildtype; (2) reduced staining levels of RB which may be incorrectly interpreted as absent; and (3) difficulties inherent in the reproducibility of scoring immune staining, especially since the intensity of RB nuclear staining may normally fluctuate throughout the cell cycle (Mittnacht & Weinberg, 1991; Xu *et al.*, 1991b).

To establish the frequency of RB inactivation in our lung cancer cell lines we have prepared protein lysates from 171 samples derived from patients with differing histologic subtypes. Using immunoblotting techniques, we defined RB inactivation as either the absence of detectable protein or the synthesis of an RB product that is either truncated or that exhibits defective phosphorylation. Our data confirms the high frequency of RB inactivation in SCLC which suggests that loss of RB activity is an essential step in the tumorigenicity of this malignancy. These data also demonstrate that the synthesis of a stable, mutant RB product is frequent in both SCLC and NSCLC tumors which will require the further development of conformation-specific antisera for large scale immune staining projects. In addition, we have correlated RB status with patient characteristics, clinical outcomes including best response to therapy and overall survival, and with *in vitro* sensitivity by MTT assays (Mosmann, 1983) to different chemotherapeutic agents.

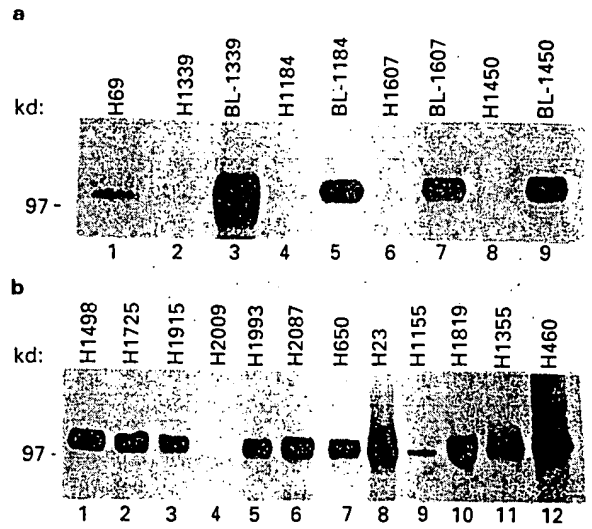

Figure 1 (a) Immunoblot analysis for RB protein expression in SCLC. Protein extracts were harvested from five SCLC samples showing mutant, hypophosphorylated protein (lane 1) or absent protein (lanes 2,4,6,8) and compared with the wildtype pattern showing multiple phosphorylated RB species from immortalized B-lymphocytes (BL) obtained from the same SCLC patients (lanes 3,5,7,9). Protein size markers are shown in kilodaltons (kD). (b) Immunoblot analysis for RB protein expression in NSCLC. Protein extracts were harvested from 12 NSCLC samples showing phosphorylated wildtype protein (lanes 1–3, 5–8 and 10–12), mutant hypophosphorylated protein (lane 9), and absent protein (lane 4)

Results

We harvested whole cell protein lysates from 166 lung cancer and five extrapulmonary small cell carcinoma (EPSC) cell lines that were in exponential growth, and scored RB protein expression as wildtype if a mobility pattern on SDS–PAGE immunoblotting showed differentially phosphorylated species migrating as closely spaced bands at approximately 110 kDa. Hypophosphorylated RB that migrates as a single band at either 110 kDa or at a truncated position secondary to an internal deletion was scored as mutant, and an undetectable signal on immunoblot analysis was scored as absent. As a control for wildtype RB protein, we examined lysates extracted from immortalized lymphocytes obtained from patients with lung cancer which was compared with the absent signal on immunoblot analysis observed with the majority of matched SCLC samples (Figure 1a). In contrast, most NSCLC samples demonstrated a wildtype RB pattern although several lanes also demonstrated either absent or mutant hypophosphorylated RB (Figure 1b). Since RB can normally exhibit a hypophosphorylated pattern in terminally differentiated cells (Chen *et al.*, 1989), after senescence (Futreal & Barrett, 1991), and in the resting phase (Go) of the cell cycle (Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989), all samples with a hypophosphorylated pattern were repeated twice to assure that cells were in exponential growth at the time that proteins were harvested. Molecular cloning of the *Rb* gene from these samples with aberrant phosphorylation have shown a mutation within the *Rb* open reading frame in every case that has been investigated to date validating this analysis (Horowitz *et al.*, 1990; Kaye *et al.*, 1990) (Kratzke, Coxon and Kaye, unpublished data). We observed absent or mutant RB in 66/75 SCLC cell lines, in 12/80 NSCLC, in 0/5 mesothelioma, in 1/6 carcinoid, and in 4/5 EPSC (Tables 1 and 2).

Since the transforming proteins of human papillomavirus (HPV), of simian virus 40 (SV40) and of adenovirus have been demonstrated to interrupt RB functional activity by competitive protein binding (Weinberg, 1991), we also examined by Southern blot analysis and polymerase chain reaction (PCR)-amplification using consensus oligonucleotides (Schiffman *et al.*, 1991) whether the tumor samples with wildtype RB protein expression had evidence for stable integration of these DNA tumor virus. We detected integration of HPV-18 DNA sequences in the single EPSC tumor cell line with wildtype RB that was obtained from a small cell carcinoma of the cervix, but did not find evidence of HPV, SV40, or adenoviral integration in other non-cervical tumor samples (data not shown).

Since surgical resection is not recommended in the clinical management of SCLC, primary tumor tissue is generally unavailable for genetic or protein analyses. Genomic DNA from several SCLC tumors, however, has been previously examined by Southern blot analysis (Harbour *et al.*, 1988) and the frequency of structural abnormalities within the *Rb* gene was similar to data obtained from the cell line analyses. In addition, we observed that when DNA was available from matched primary tumor tissue and from the corresponding derived cell line the results were concordant, even after the cell line had been in continuous culture for greater than two years (Figure 2). We also noted concordance of RB protein status when cell lines were generated from the same patient at different stages in the course of the lung malignancy (SCLC lines with absent RB expression: H2059,2141; NSCLC lines with

wildtype RB expression: H1993/H2077) as well as when derived cell lines and matched lines from transplated nude mouse xenografts were compared (SCLC lines with mutant RB carrying a deletion of exon 22: H249/N592). Further, we observed that the incidence of RB inactivation in our sample of 80 NSCLC cell lines (15%) was lower than that recently reported in a series of primary NSCLC tumors that utilized an immunohistochemical technique (30–35%) (Xu *et al.*, 1991a; Reissmann *et al.*, 1993), again suggesting that the *Rb* mutations detected are not a consequence of cell culture propagation.

Retrospective analysis of corresponding clinical data using Fisher's exact test showed no statistically significant associations between RB protein inactivation and age, sex, extent of disease, performance status, smoking history, previous treatment, and best clinical response in SCLC (Table 3) and NSCLC (Table 4) patients. In addition, we observed no consistent correlation of RB protein expression with overall survival

Table 1 RB protein status

	Wild type	*Mutant*	*Absent*	*Total*
SCLC	9 (12%)	12 (16%)	54 (72%)	75
Non-SCLC	68 (85%)	3 (4%)	9 (11%)	80
Carcinoid	5 (83%)	1 (17%)	0 (0%)	6
Mesothelioma	5 (100%)	0 (0%)	0 (0%)	5
EPSC	1 (20%)*	1 (20%)	3 (60%)	5

*HPV-18 positive

Table 2 RB protein expression in lung cancer cell lines

Small cell lung cancer				*Non-small cell lung cancer*			
H60	–	H1836	–	H23	+	H1693	+
H82	–	H1882	–	H125	+	H1703	+
H123	–	H1963	–	H226	+	H1717	+
H128	–	H2028	–	H322	+	H1725	+
H187	–	H2029	–	H358	+	H1755	+
H196	–	H2059	–	H441	+	H1792	+
H250	–	H2081	–	H460	+	H1819	+
H289	–	H2106	–	H520	+	H1824	+
H345	–	H2107	–	H522	+	H1838	+
H372	–	H2171	–	H640	+	H1915	+
H378	–	H2141	–	H647	+	H1930	+
N417	–	H2330	–	H650	+	H1944	+
H433	–	H2332	–	H661	+	H1993	+
H524	–	H69	mt	H676	+	H2030	+
H526	–	H146	mt	H726	+	H2077	+
H620	–	H209	mt	H810	+	H2086	+
H678	–	H249	mt	H820	+	H2087	+
H689	–	H298	mt	H838	+	H2122	+
H735	–	N592	mt	H854	+	H2126	+
H738	–	H748	mt	H1264	+	H2145	+
H740	–	H1045	mt	H1299	+	H2170	+
H774	–	H1238	mt	H1334	+	H2250	+
H862	–	H1436	mt	H1355	+	H2286	+
H889	–	H1618	mt	H1373	+	H2347	+
H930	–	H2227	mt	H1385	+	H2369	+
H1059	–	H211	+	H1395	+	H2405	+
H1062	–	H360	+	H1404	+	H2409	+
H1092	–	H446	+	H1435	+	H2427	+
H1105	–	H450	+	H1466	+	H1155	mt
H1173	–	H719	+	H1498	+	H2172	mt
H1184	–	H792	+	H1563	+	H2712	mt
H1284	–	H841	+	H1568	+	H596	–
H1304	–	H865	+	H1570	+	H969	–
H1315	–	H1522	+	H1573	+	H1710	–
H1339	–			H1581	+	H1734	–
H1450	–			H1623	+	H1781	–
H1514	–			H1648	+	H2009	–
H1607	–			H1650	+	H2066	–
H1648	–			H1651	+	H2228	–
H1628	–			H1666	+	H2527	–
H1672	–						

Absent (–), mutant (mt), and wild type (+) RB protein expression

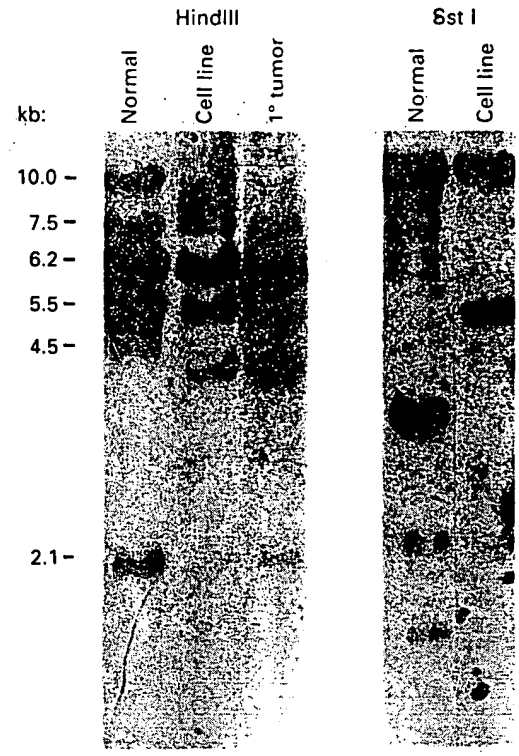

Figure 2 DNA blot analysis of *Rb* gene in matched normal tissue, primary tumor, and derived cell line. 10 μg genomic DNA was extracted from normal tissues (normal), primary tumor (1° tumor), and derived cell line (cell line) and cut to completion with the restriction enzymes HindIII and SstI. Hybridization was conducted using the partial cDNA probe p3.8r (Friend *et al.*, 1986) under conditions described in Materials and methods

Table 3 Characteristics of SCLC patients

	RB protein expression		
Characteristic	*Wild type*	*Altered*	*P-value*
Age			
20–49	0	11	1.0
50–59	5	21	(20–59 *vs* 60+)
60+	3	20	
Male:female	4:4	38:15	0.36
Extent of disease[a]			0.37
Limited	3	11	
Extensive	5	41	
Performance status[b]			0.69
0–1	5	37	
2–4	3	15	
Smoking (pack-years)			0.56
1–30	0	6	
31+	4	20	
Best response[c]			1.0
CR	3	17	(CR/PR *vs* other)
PR	4	26	
NR	1	2	
PD	0	4	
NE	0	3	

[a]Limited: all lung tumor encompassed within a tolerable radiation therapy port; extensive: known tumor outside of a radiation therapy port. [b]Performance status: 0-normal activity; 1-symptomatic but ambulatory; 2-bedridden <50% of waking hours; 3-bedridden >50% of waking hours; 4-completely bedridden. [c]CR-complete response; PR-partial response (>50% reduction in the sum of the areas of all tumor lesions); NR-no response; PD-progressive disease; NE-not evaluable

measured from the date of diagnosis) using the Mantel-Haenszel technique to determine the significance of difference between pairs of corresponding Kaplan-Meier survival curves (Kaplan & Meier, 1958; Mantel, 1966) (Figure 3).

Table 4 Characteristics of NSCLC patients

Characteristic	RB protein expression: Wild type	RB protein expression: Altered	P-value
Age			0.05
20–49	15	3	(20–59 *vs* 60+)
50–59	22	1	
60+	10	5	
Male:female	42:20	9:5	1.0
Stage			
1	4	1	1.0
2	3	0	(1–2 *vs* 3–4)
3A	13	2	
3B	8	0	
4	18	6	
Performance status			
0–1	37	10	0.43
2–4	13	1	
Smoking (pack-years)			
1–30	10	3	0.36
31+	25	3	
Histology			
adeno	38	7	0.53
adenosquamous	2	1	
large cell	14	3	
squamous	6	0	
Best response			
CR	1	0	1.0
PR	5	1	(CR/PR *vs* other)
NR	7	2	
PD	13	3	
NE	20	2	

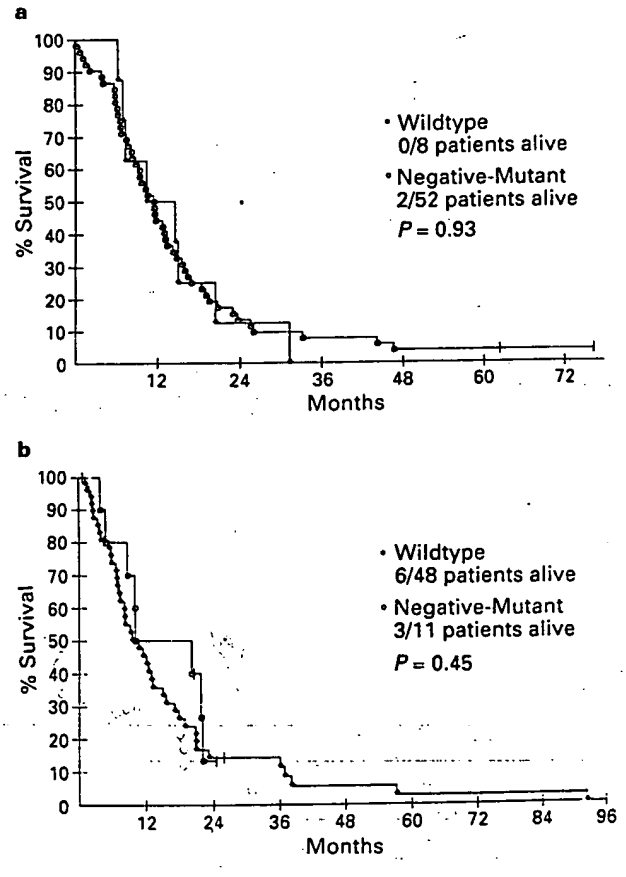

Figure 3 (a) Effect of RB status on overall survival in SCLC. (b) Effect of RB status on overall survival in NSCLC

Since RB inactivation was less prevalent in NSCLC, mesothelioma, and carcinoid samples which generally represent tumors with an *in vivo* chemotherapy-resistant phenotype, we examined whether RB status predicted *in vitro* drug sensitivity to three commonly used chemotherapeutic agents for lung cancer: etoposide (VP-16), cisplatin (CDDP), and doxorubicin (DOX). The *in vitro* drug sensitivity testing was obtained using the quantitative MTT [(3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983) and was performed immediately after the tumor procurement as part of an approved clinical protocol for NSCLC. The retrospective comparison of the IC_{50} data (the drug concentration that results in a 50% inhibition of cell growth), however, showed no differences between samples with wildtype (wt) and abnormal (abn) RB expression (Figure 4). To directly address the effect of RB expression on *in vitro* drug sensitivity we transfected two different RB(−) SCLC cell lines (H187 and N417) with an RB expression vector under the control of a cytomegalovirus (CMV) promoter as previously published (Kaye *et al.*, 1990; Kratzke *et al.*, 1993). We confirmed the stable expression of RB protein in the expanded transfectants (data not shown) and tested mock and RB-transfected cells for drug sensitivity by the MTT assay for either etoposide (VP-16; concentration range: 0–25 μM), cisplatin (CDDP; range: 0–120 μM), or doxorubicin (Dox; range 0–8 μM). In this analysis, which represents three independent experiments using 8 microwell replicates for each drug exposure, we observed a trend toward increased drug resistance to the cytotoxic agents in two of the three independent RB transfectants from the H187 SCLC cells (Figure 5) and in both RB transfectants from the N417 SCLC cells (Figure 6).

Discussion

This study extends the initial reports of RB inactivation in SCLC and demonstrates that approximately

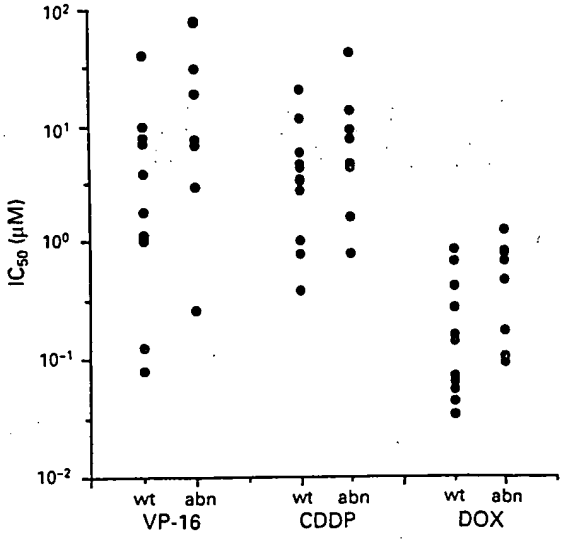

Figure 4 Retrospective analysis of the effect of RB status (wild-type, wt, or abnormal, abn) on the *in vitro* drug sensitivity testing in NSCLC cell lines for etoposide (VP-16), cisplatin (CDDP), and doxorubicin (DOX). The IC_{50} designates the drug concentration resulting in 50% cell inhibition using the MTT assay

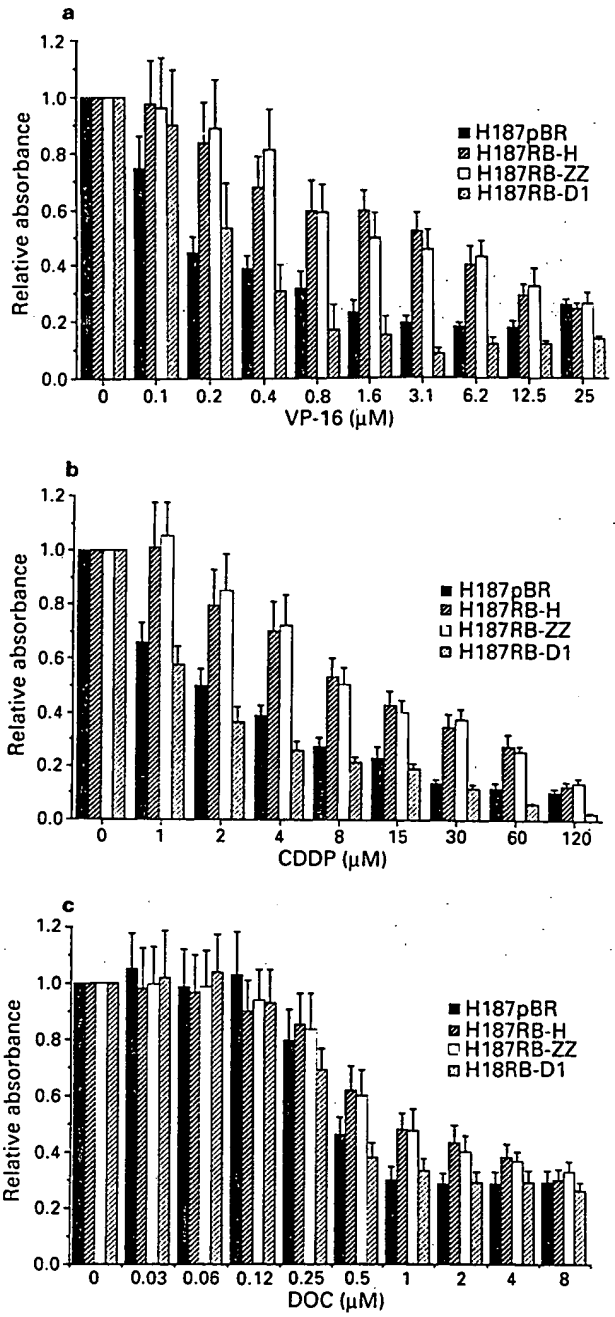

Figure 5 Drug sensitivity testing of mock (pBR) and RB-transfected (clones H,ZZ,D1) H187 cells using the MTT assay. (a) etoposide (VP-16); (b) cisplatin (CDDP); (c) doxorubicin (DOX)

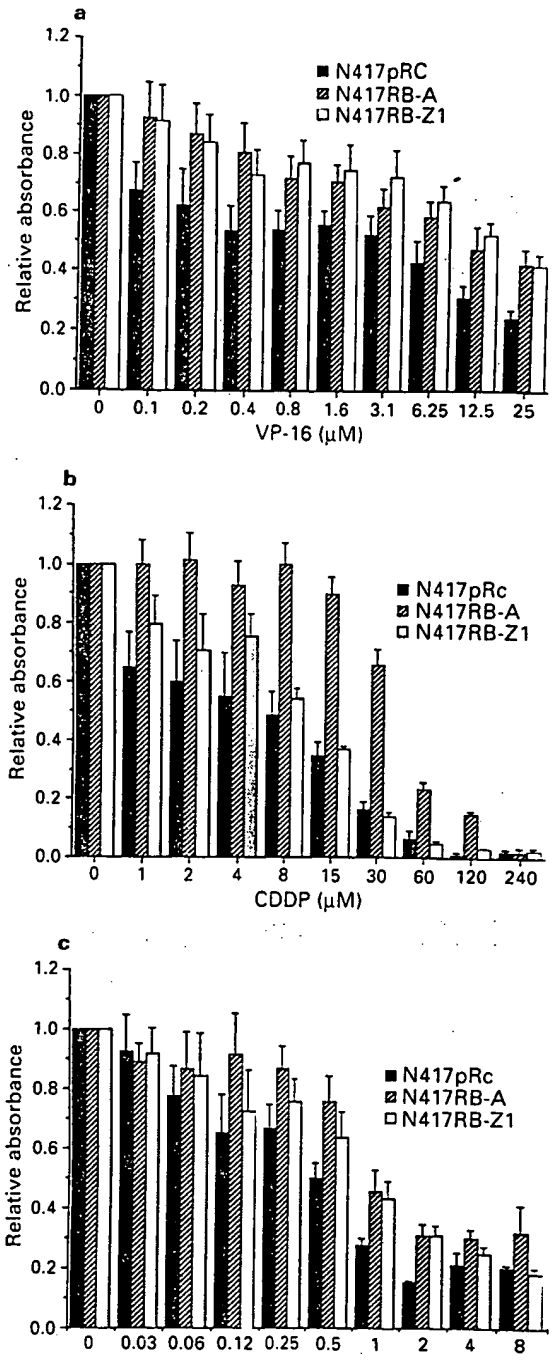

Figure 6 Drug sensitivity testing of mock (pRC) and RB-transfected (clones A,Z1) N417 cells using the MTT assay

90% of these tumors have either absent or aberrant expression of the RB product. This observation raises several important points. First, it suggests that loss of RB activity is a necessary step for the development of small cell carcinoma of the lung, and possibly also for small cell carcinomas that arise in extrapulmonary sites such as the brain, prostate and cervix. Why the incidence of RB inactivation in small cell carcinoma resembles the mutation rates observed in retinoblastoma is still unknown, and underscores the importance of attempting to identify the cell of origin of SCLC to study its patterns of gene expression. This finding, however, also raises the question of why several SCLC tumors still retain wildtype RB expression. One possible explanation is that these tumors were removed from patients who presented with undifferentiated lung tumors that were misdiagnosed as SCLC, and in at least one case (H841) the morphologic and biochemical features of this cell line more closely resemble characteristics observed with NSCLC. Another possible explanation is that these tumors may have inactivated the RB tumor suppressor pathway either by infection with a DNA tumor virus or by mutational inactivation of a member of the growing family of modulating RB binding proteins. With the exception of a small cell carcinoma of the cervix that was HPV-18 positive (H1341), however, we have not documented evidence of infection with DNA tumor viruses in any of the other samples with wildtype RB expression. In addition, we have not observed abnormalities with the

expression of several cellular RB binding proteins (Otterson *et al.*, 1993; Kim & Kaye, unpublished data) in these cell lines. Recently, mutational inactivation of a cyclin dependent kinase (cdk) inhibitor, designated p16, has been observed in a wide range of human tumors including lung carcinomas (Kamb *et al.*, 1994; Nobori *et al.*, 1994). Since p16 appears to selectively target a kinase activity that may be specific for RB phosphorylation (Serrano *et al.*, 1993), it remains possible that loss of p16 activity may be an alternate mechanism to disrupt RB tumor suppressor pathway in these tumors with wildtype RB expression.

In contrast to SCLC, most NSCLC tumors (approximately 85%) express wildtype RB protein. This finding highlights the distinct genetic etiology of SCLC and NSCLC tumors, despite both presenting as tobacco-related lung malignancies. Previous studies have investigated the incidence of RB inactivation in primary NSCLC tumor tissue using immunohistochemical analysis and observed abnormalities in approximately 30–35% of the samples tested (Xu *et al.*, 1991a; Reissmann *et al.*, 1993). Our finding of a lower incidence of RB mutations in NSCLC cell lines than that detected using primary tumor tissues suggests that RB abnormalities are not selected for by cell culture propagation and that the detection of absent or aberrant RB protein by immunoblot analyses may be more specific and reproducible than current immunohistochemical techniques. These observations of a lower incidence of RB abnormalities in NSCLC than in SCLC are also consistent with epidemiological data on non-ocular tumors in retinoblastoma survivors or in the immediate family members of patients with familial retinoblastoma. These studies have suggested a higher incidence of lung tumors in the younger patients (Strong *et al.*, 1984) and the 10-fold increase in lung tumors observed in these families was predominantly of the small cell ('oat cell') histology (Sanders *et al.*, 1989).

We also examined whether the presence of RB inactivation was a prognostic marker for clinical outcome in lung cancer. Analysis of RB status in 60 SCLC and 59 NSCLC samples for which we had matched clinical data did not reveal a correlation with patient characteristics or with response to therapy and overall survival. This finding is in contrast to previous studies in malignant sarcoma (Cance *et al.*, 1990) and with carcinoma of the bladder (Cordon-Cardo *et al.*, 1992; Logothetis *et al.*, 1992) which suggested that RB alterations are associated with an adverse outcome, but is similar to a Lung Cancer Study Group analysis which also failed to detect a significant correlation with RB status and survival (Reissmann *et al.*, 1993). This type of comparative study, however, is limited by the small numbers of SCLC samples with a wildtype RB phenotype and, conversely, by the relatively small number of NSCLC samples with RB inactivation. More importantly, the overall poor response of most lung cancers to available treatments may mask important biologic differences which might still exist between RB(+) and RB(−) SCLC and NSCLC tumors.

We also examined the effect of RB expression on drug resistance to a variety of commonly used chemotherapeutic agents. Although a retrospective analysis did not show any significant difference in the IC_{50} of etoposide, cisplatin and doxorubicin between RB(+) and RB(−) NSCLC samples, we observed that the stable expression of RB in two different SCLC cell lines resulted in a modest increase in drug resistance in four out of five independent transfectants as compared to mock-transfected cells. These differences may reflect RB-mediated delays in the transit time through the G1 phase of the cell cycle (Goodrich & Lee, 1992), however additional work is needed to define this possibility and to determine the influence of p53 mutations that are frequently present in these cells as well.

In summary, inactivation of the RB tumor suppressor pathway appears to be an essential step for the development of SCLC. p53 is the only other gene that has been shown also to be a consistent target for somatic mutations in this tumor (Takahashi *et al.*, 1989; Kratzke *et al.*, 1992). Interestingly, reintroduction of either p53 (Takahashi *et al.*, 1992) or RB (Kratzke *et al.*, 1993; Ookawa *et al.*, 1993) into RB(−) lung cancer cells has been associated with either growth arrest or partial suppression of tumorigenicity. This suggests that inactivation of both genes is required in SCLC and parameters of tumorigenicity can then be suppressed by complementation with either wildtype gene. The ongoing characterization of these and other candidate tumor suppressor genes that may act as rate-limiting determinants for the development of lung cancer, such as the recent p16 gene (Kamb *et al.*, 1994; Nobori *et al.*, 1994), remains an important goal toward the design of new therapeutic strategies.

Materials and methods

Patients and cell lines

Tumor samples from patients with histologically confirmed primary or metastatic SCLC, EPSC, NSCLC, pulmonary carcinoid, and mesothelioma were obtained as part of standard diagnostic, staging, or therapeutic procedures following appropriate informed consent. 171 cell lines were established using previously described culture techniques (Carney *et al.*, 1985) and propagated in RPMI media supplemented with 10% fetal bovine serum and antibiotics. Data regarding clinical parameters and survival from 60 SCLC and 59 NSCLC patients were available from updated computerized records for this study.

Drugs and chemicals

Cisplatin (CDDP) and etoposide (VP-16) were purchased from Bristol Laboratories, Inc., Syracuse, NY. Doxorubucin (DOX) was purchased from Adria Laboratories, Inc., Columbus, OH. DOX, CDDP, and VP-16 were dissolved in distilled water and diluted with phosphate-buffered saline (PBS) before use. 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and G-418 were purchased from Sigma Chemical Co., St. Louis, MO. Media and supplements for cell culture were obtained from Gibco, Grand Island, NY.

Immunoblotting

Cellular protein was extracted by lysing 5×10^6 cells in 1 ml of 50 mM Tris-HCl, pH 7.5/250 mM NaCl/5 mM EDTA/0.1% Nonidet P-40 (NP-40)/50 mM NaF/1 mM phenylmethylsulfonyl fluoride (PMSF) containing leupeptin at $10\,\mu g\,ml^{-1}$ and aprotinin at $50\,\mu g\,ml^{-1}$, followed by quick-freezing of the supernatant. Seventy-five micrograms of cellular protein was electroblotted to nitrocellulose paper after separation on 7.5% SDS–PAGE and then allowed to incubate overnight at 4°C with a 1:100 dilution of monoclonal anti-RB antibody

(PharMingen, San Diego). The immunoblot was subsequently incubated for 4 h with a 1:200 dilution of polyclonal rabbit anti-mouse immunoglobulin antibody (PharMingen), followed by a 2 h incubation with 2.5×10^6 c.p.m. of ^{125}I-labeled protein A and autoradiography.

DNA blot analysis

10μg of genomic DNA was extracted and subjected to restriction endonuclease analysis as previously described (Davis *et al.*, 1986). The p3.8r *Rb* cDNA probe (Friend *et al.*, 1986) was random-primed with [^{32}P]CTP and hybridization and washing conditions were as previously described (Davis *et al.*, 1986).

Stable expression of Rb *gene in RB(−) small cell lung cancer cell lines*

Eukaryotic expression vectors containing the wild-type RB open reading frame and encoding neomycin resistance were transfected via lipofection as described by the manufacturer (Life Technologies) into the RB(−) SCLC cell lines H187 and N417. G418-resistant single clones were expanded, and subjected for immunoblotting and MTT assay.

MTT assay

To exclude the effect of G418 in medium, at least 3 days before MTT assay all cell clones were washed three times and resuspended in RPMI containing 10% fetal bovine serum. Single-cell suspensions were prepared by mechanical disaggregation of SCLC cell lines. The MTT assay was performed as previously described (Carmichael *et al.*, 1987). Briefly, after the cell count with a hemacytometer, cells were incubated in 180 μl of medium for 24 h using 96 well multiplates (Costar, Cambridge, MA) prior to the drug addition. Then 20 μl of 10× drug solution was added to each well. Following a 4 day incubation, 100 μg of MTT was added to each well and incubated for 4 h. The plates were then centrifuged at 1000 *g* for 5 min. The supernatant was removed by aspiration and 150 μl of 100% DMSO was added to each well to resolubilize the MTT formazan crystal. The spectophotometric absorbance at 560 nm was determined using a scanning multiwell spectrophotometer (Biotek Instruments Inc., Burlington, VT). Dose-response curves were plotted for all drugs with the 50% inhibitory concentration (IC_{50}) for each cell line and represented graphically as the dose of drug causing a 50% reduction in absorbance compared to control values. In the experiment to evaluate the chemosensitivity of the mock and RB-transfected cells, the MTT assay was repeated at least three times using eight replicate samples each time.

Statistical analysis

The Kaplan-Meier method was used to estimate the probability of survival as a function of time (Kaplan & Meier, 1958), and the Mantel-Haenszel method was used to assess the significance of the difference between pairs of survival probabilities (Mantel, 1966). Survival was calculated from the date of diagnosis until the date of death or date of last follow-up. All reported P values are two sided.

Note added in proof

Xu *et al.* (1994) recently reported that altered RB protein was associated with decreased survival in early stage non-SCLC.

Acknowledgements

We are grateful to Lynn Hankins and Ruby Phelps for their expert assistance in the data management of patients entered on lung cancer protocols.

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[CANCER RESEARCH 56, 3199–3202, July 15, 1996]

Advances in Brief

Reversible, p16-mediated Cell Cycle Arrest as Protection from Chemotherapy

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Abstract

A model system has been developed to explore the relationship between cell cycle arrest and chemotherapeutic toxicity. An isopropyl-1-thio-β-D-galactopyranoside-inducible *P16* construct was introduced stably into a melanoma cell line and used to promote G_0-G_1 arrest in the recipient cells. The state of arrest was reversible and did not compromise cell viability over a period of at least 7 days. Isopropyl-1-thio-β-D-galactopyranoside-treated, arrested cells were significantly more resistant to the chemotherapeutic agents methotrexate (~50 times), vinblastine (>100 times), and cisplatin (~10 times) compared to controls. This strategy of protection from chemotherapy exploits one of the basic genotypic differences between normal cells and tumor cells: the integrity of genetic pathways that regulate growth.

Introduction

For the past several decades, radiation and chemical toxins designed to kill dividing cells have been the basis for cancer therapy. Although these anticancer agents may destroy tumor cells, they may also kill cycling nontumor cells such as intestinal epithelia, hair follicle cells, and hematopoietic precursors. These side effects have serious consequences. Side effects including cachexia (appetite loss), alopecia (hair loss), nausea, and immunosuppression limit the dose of anticancer agents. Partly for this reason, some tumor cells inevitably escape treatment. These surviving cells may give rise to tumors that are resistant to drug or radiation therapy. Despite advances in anticancer regimens, systemic toxicity and postsurgery tumor relapse remain challenging problems.

In principle, the deleterious effects of conventional cancer therapy would be diminished if nontumor cells were protected from cytotoxicity (1–4). Fewer normal cells would die and, since larger drug doses could be administered, fewer cancer cells would survive. Actively dividing normal cells might be protected from cancer therapy if they were induced deliberately to enter cell cycle arrest (1–4). In a quiescent state, cells are likely to be more resistant to the damaging effects of cancer therapy. However, for this approach to succeed, several conditions must be satisfied: (*a*) normal cells must arrest growth, whereas tumor cells do not; (*b*) the arrested cells must remain viable; and (*c*) the arrest must be reversible. These conditions might be achieved by taking advantage of the inherent genetic differences between normal and tumor cells. Genetic pathways that regulate cell growth in normal cells are inactivated by mutation in tumors. As a result, appropriately directed induction of these pathways should have no effect on tumor cells while causing normal cells to arrest. For instance, activation of the p16/Rb pathway by ectopic expression of p16 is sufficient to cause cell cycle arrest in a variety of Rb+ cell types but not in Rb− tumor cells (5, 6). Genetic and biochemical studies suggest that this pathway regulates growth in a wide variety of cell types and is frequently inactivated during tumor progression *in vivo* (7, 8). Therefore, the p16/Rb pathway is ideal for exploring the feasibility of cellular protection through reversible cell cycle arrest.

Materials and Methods

Cell Lines. H294T is a melanoma cell line in which *P16* is deleted homozygously (data not shown). This cell line was used as the parent for construction of a line in which p16 expression is induced by IPTG[2] using the lac-switch technology as described by the manufacturer (Stratagene). Briefly, the cell line HS294T was transformed with the lac I expression vector (p3'SS; Stratagene) by electroporation. Transformants were selected in medium containing 300 μg/ml hygromycin B (Boehringer Mannheim). Twelve transformed cell lines were cloned and assayed for lac repressor expression by reverse transcription-PCR. A cell line expressing relatively large amounts of the transgene was chosen for additional experiments (designated HS294T/laci). HS294T/laci was transformed with p16-containing expression vector (pOPRSVI.p16) by electroporation. pOPRSVI.p16 was constructed by cloning the entire p16 coding sequence into the *Not*I site of pOPRSVICAT (Stratagene). The p16 initiation codon was modified to match a consensus Kozak initiation sequence (9). Transformed cells were selected in media containing hygromycin B and 300 μg/ml Geneticin (Life Technologies, Inc.). Twelve independent cell lines were isolated, of which six were subsequently shown to undergo clear cell cycle arrest after treatment with IPTG. One of these cell lines, designated HS294T/*P16*+, was chosen for additional experiments. HS294T/laci was also transformed with pOPRSVICAT expression vector to create HS294T/*P16*- control cell lines. Three independent cell lines of this type were isolated and used as negative controls in experiments described in this study. All cell lines were grown in DMEM media (BioWhittaker), supplemented with 10% fetal bovine serum, nonessential amino acids, and sodium pyruvate.

Western Analysis. Approximately equal amounts of total cellular protein were resolved on 15% SDS PAGE gels. After electrophoresis, proteins were transferred to Mylar membrane as described by the manufacturer (Hoefer Scientific). p16 protein was detected using an anti-p16 monoclonal antibody (kindly provided by Gordon Peters, Imperial Cancer Research Fund Laboratories London, United Kingdom) and a goat anti-mouse biotinylated secondary antibody (Vector).

Drug Resistance. Equal numbers of HS294T/*P16*+ cells were plated, and one-half of the samples were pulsed with IPTG at $t = 0$ for 12 h. At $t = 24$, all of the samples (IPTG-treated and untreated) were pulsed for 12 h with the indicated chemotherapeutic agent at the indicated concentration. The percentage of viability was estimated by comparing the number of viable cells present at each drug concentration to the no-drug control. Viable cells were identified by exclusion of 0.4% trypan blue. HS294T/*P16*− cells were treated identically in control experiments.

Results and Discussion

The melanoma-derived cell line HS294T, in which *P16* is homozygously deleted, was engineered to contain *P16* under the regulation of *Escherichia coli* lac sequences. In this construct, *P16* gene expression is normally repressed but can be induced by the addition of IPTG to the growth medium. Six such cell lines were derived from the parental HS294T cell line. All six lines ceased to grow in the presence of IPTG, whereas the parental line (or lines with an IPTG-inducible promoter driving CAT expression) continued to proliferate. One of these inducible lines, termed HS294T/*P16*+, was chosen for subse-

Received 3/13/96; accepted 5/29/96.

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[2] The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; CAT, chloramphenicol acetyltransferase; Rb, retinoblastoma.

quent experiments. Flow cytometry revealed that HS294T/*P16+* was arrested at the G_0-G_1 stage of the cell cycle in the presence of IPTG (Fig. 1), and two independently derived HS294T/P16+ cell lines showed similar IPTG-induced arrest characteristics (Table 1). HS294T/P16+ is, in our model system, equivalent to *P16+* normal cells; derivatives of the parental line without the p16 expression vector are analogous to *P16−* tumor cells.

The response of HS294T/*P16+* to IPTG was examined in two additional ways: (*a*) different doses of IPTG were applied to the cells for 12 h. The percentage of cells in G_0-G_1 was measured to determine a sufficient dose to cause cell cycle arrest (data not shown). This dosage, 0.1 mM, was used for all subsequent experiments; and (*b*) immunoblots using anti-p16 antibody were used to demonstrate that the addition of IPTG to HS294T/*P16+* cells caused increased p16 protein expression (Fig. 2). Cell lysates were prepared from HS294T/*P16+* and *P16−* cells after they had been exposed to IPTG for 24 h. HS294T/*P16−* cells produced undetectable levels of p16 protein. In contrast, IPTG-treated HS294T/*P16+* cells produced easily detectable amounts of p16 protein, approximately equal to the level of p16 expression in the Rb-negative cell line 5637.

To determine if p16-induced arrest was reversible, HS294T/*P16+* cells were pulsed with IPTG for 12 h. After the IPTG was removed, the cells were monitored by flow cytometry, and the percentage of cells in G_0-G_1 as a function of time following IPTG removal was

Table 1 *Response of P16+ cell lines to IPTG*

Cell line	% G_0-G_1[a] −IPTG	+IPTG
HS294T/P16+	49	84
HS294T/P16+(B)	68	94
HS294T/P16+(C)	62	95
HS294T/CAT (control)	51	56

[a] Response was measured by flow cytometry 36 h after the addition of IPTG to the cell culture medium.

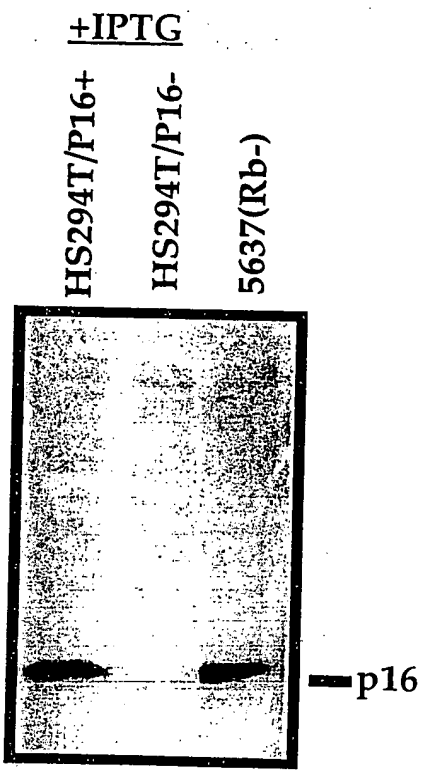

Fig. 2. Expression of p16 protein as a result of IPTG treatment. After 24 h exposure to IPTG, roughly 10^7 cells from the indicated cell line were harvested and lysed for Western analysis. The Rb-negative (and hence p16-overexpressing) cell line 5637 served as the positive control.

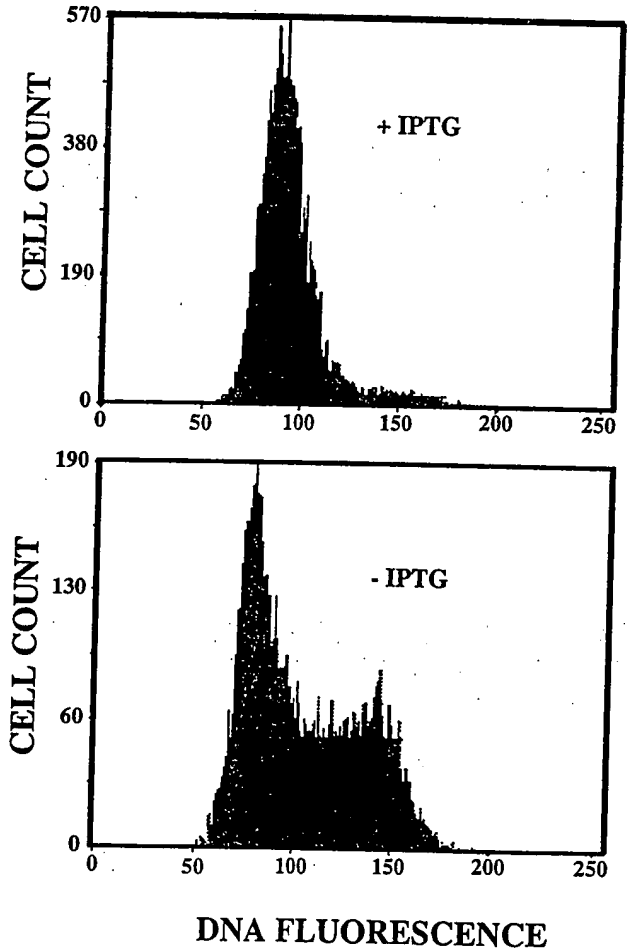

Fig. 1. Cell cycle arrest induced by IPTG. Distribution of cells with 2N DNA content (G_0-G_1 peak at 75 fluorescence units) and 4N content (G_2 peak at 150 fluorescence units) after treatment of H294T/*P16+* cells with IPTG as compared to non-IPTG treated controls. In the IPTG-treated cells, roughly 90% of the cells are in G_0-G_1 as compared to ~50% for the untreated sample. The analysis was conducted on a flow cytometer (Becton Dickinson) after fixation in ethanol and staining with propidium iodide.

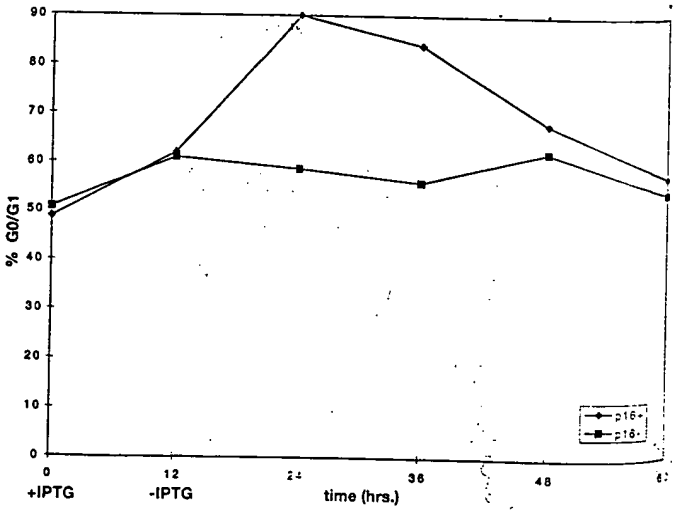

Fig. 3. Reversibility of p16-induced cell cycle arrest. Graph of the percentage of cells in G_0-G_1 (*Y axis*) *versus* time (*X axis*). *p16+*, HS294T/*P16+* cell line treated with IPTG. *p16−*, HS294T/*P16−* cell line treated with IPTG. The results represent the mean of three experiments. Both cell lines were pulsed for 12 h with 0.1 mM IPTG. IPTG was added at $t = 0$ and removed at $t = 12$. Cells were harvested at 12-h time intervals, starting at $t = 0$. After harvesting, the cells were fixed in ethanol and analyzed by flow cytometry.

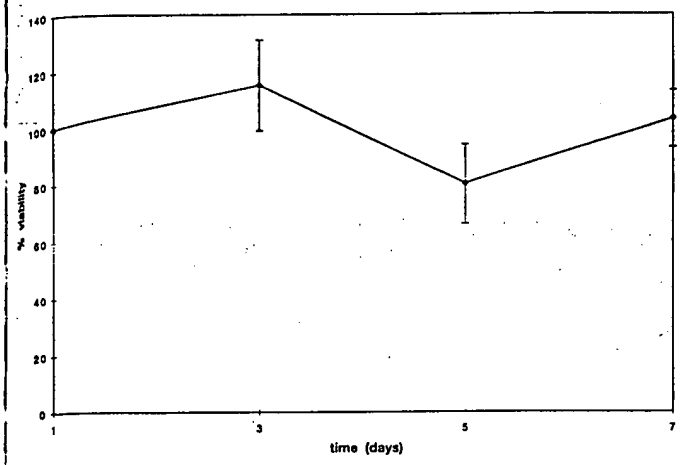

Fig. 4. Viability of cells as a function of time spent in arrest. Graph of the viable cells present at the indicated day as a percentage of the number present at day 1 (*Y axis*) *versus* time spent in IPTG (*X axis*). Cell cultures containing the same number of HS294T/*P16+* cells were treated with 0.1 mM IPTG at $t = 0$. At each time point, the cells from a single culture were harvested and counted on a hemocytometer. Viable cells were identified by exclusion of 0.4% trypan blue. The cells were fed with fresh medium every 4 days. The results represent the mean of three experiments; *bars*, SD.

determined (Fig. 3). These results indicated that HS294T/*P16+* cells were completely arrested 24 h after exposure to IPTG and remained so for another 12 h. However, by 48 h, the number of cells in G_0-G_1 returned to levels characteristic of the normal state of growing cells (50–60%). The number of G_0-G_1 cells remained relatively constant in the *P16−* control cell line treated identically. In addition, cells that had been arrested by p16 induction and then released could be arrested again by an additional dose of IPTG (data not shown). These data strongly suggest that p16-induced arrest is reversible in a majority of the cells. Furthermore, the reversal is rapid.

To analyze cell viability as a function of the duration of cell cycle arrest, HS294T/*P16+* cells were left in IPTG for several days. The number of cells present before IPTG treatment was compared to the number of cells present at various time points after treatment with IPTG (Fig. 4). Cell viability remained high even after 7 days in the presence of IPTG. To ensure that the cells were capable of reentering the cell cycle and were not trapped in a permanent state of arrest, cells were treated with IPTG for 6 days, after which the IPTG was washed away, and the cells were monitored by flow cytometry. At the time the IPTG was removed, about 90% of cells were in G_0-G_1, but after 4 days without IPTG, the percentage of G_0-G_1 cells had dropped to normal levels (data not shown). These results suggest that artificially induced expression of p16 is not lethal for H294T cells in the short term. Considerable numbers of cells remain viable and able to reenter the cell cycle, even after 7 days of dormancy.

Susceptibility of H294T/*P16+* cells to chemotherapeutic agents was tested with three agents: methotrexate, vinblastine, and cisplatin. These compounds represent distinct classes of chemotherapeutic drugs, each with a different mode of action. Methotrexate inhibits DNA synthesis by blocking production of thymidine (10). Vinblastine is an alkaloid that destabilizes microtubules, disrupting the mitotic spindle (11). Cisplatin reacts with DNA to form platinated complexes that are repaired poorly within the cell (12).

HS294T/*P16+* cells were pulsed for 12 h with IPTG to induce cell cycle arrest. After 24 h, a period sufficient for nearly complete G_0-G_1 arrest, the cells were exposed to either methotrexate, vinblastine, or cisplatin. Twelve h later, the drug was removed. The time at which cell viability was assessed varied depending on the chemotherapeutic agent but was always long enough after drug treatment to ensure that

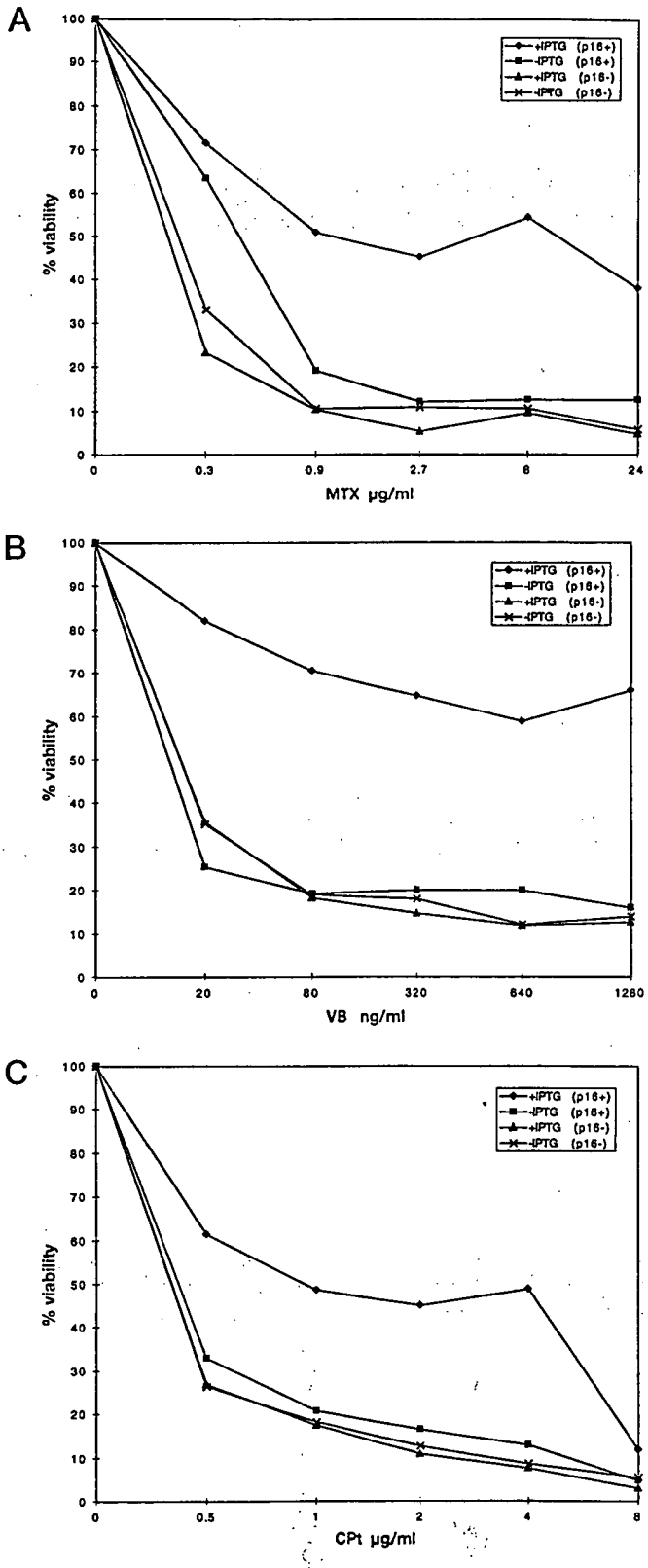

Fig. 5. Resistance of arrested cells to chemotherapeutic agents. *A*, graph of cell viability after treatment with methotrexate (*Y axis*) *versus* drug concentration (*X axis*). The number of viable cells was assessed 72 h after drug addition. *B*, vinblastine: viable cells (*Y axis*) *versus* drug concentration (*X axis*). The number of viable cells was assessed 48 h after drug addition. *C*, cisplatin: viable cells (*Y axis*) *versus* drug concentration (*X axis*). The number of viable cells was assessed 60 h after drug addition. *p16+*, HS294T/*P16+* cell line. *p16−*, HS294T/*P16−* cell line. The results represent the means of at least three experiments.

the maximum number of cells had died, and long enough after removal of IPTG to ensure that the viable cells had reentered the cell cycle. With each compound (methotrexate, vinblastine, or cisplatin), IPTG-treated HS294T/*P16+* cells were resistant compared with *P16−* cells or HS294T/*P16+* cells not exposed to IPTG (Fig. 5). For cisplatin, the difference in LD_{50}s (defined as the drug dose at which one-half the cells are dead at the time of assay as compared to the nondrug-treated controls) between the arrested cells and the growing control cells was least impressive, about 8-fold. Resistance to methotrexate was intermediate, a difference in LD_{50} of about 80-fold. For vinblastine, the difference was over 100-fold. Thus, in this model system, vinblastine displayed the most dramatic specificity for killing growing cells. This may be due to the primary cytotoxic effect of vinblastine during the G_2-M transition. Cells in G_0-G_1 arrest may be generally resistant to such activity.

The intent of these experiments was to model a therapeutic strategy based on pretreatment with a molecule that causes reversible cell cycle arrest in normal cells, followed by administration of traditional chemotherapy. In this model system, the engineered melanoma cell line enters G_0-G_1 arrest, withstands increased doses of chemotherapeutic agents, and reenters the cell cycle after the inducer is withdrawn. Several aspects of the strategy modeled here remain to be explored further. Will normal, nontransformed cells respond to p16 induction in a similar manner? It is possible that apoptotic programs may be activated by such treatment, or that terminal differentiation may ensue. Either of these responses could severely limit the utility of the method. How can p16 induction be achieved *in vivo*? In the model described here, regulation was accomplished through an artificial, IPTG-inducible construct. A specific inducer of p16 in normal cells has not been identified, although p16 expression can be increased roughly 50-fold through an unknown mechanism that may depend indirectly on Rb (13–17).

Among the strengths of the therapeutic approach modeled here is its generality. Cytotoxic effects of three anticancer agents with significantly different modes of action were ameliorated by cell cycle arrest. The most dramatic difference was observed with vinblastine, suggesting that other agents specifically directed against the G_2 or M phases might be most effective. In combination with such traditional chemotherapeutic agents, reversible arrest might increase the therapeutic window, the range of drug concentrations that separate therapeutic efficacy from systemic toxicity. A second strength is that the strategy exploits the fundamental genotypic differences between normal and tumor cells, the integrity of growth control pathways. In these experiments, the p16 regulatory pathway was targeted, a pathway that is inactivated in many tumors. Tumor cells that have lost p16 function by deletion or mutation, or the activity of any elements such as Rb which lie downstream of p16 in the genetic pathway, should be incapable of arresting in response to a molecule that induces p16 expression in normal cells (5, 6). Thus, the inducer molecule need not be delivered specifically to normal cells. Other pathways for growth control, including the p53 pathway, may also be targets for this type of strategy.

Acknowledgments

We thank Gordon Peters for the gift of the p16 monoclonal antibody and Jennifer Geary for assistance with tissue culture.

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